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(54) **METHOD OF MODIFYING A YEAST CELL
FOR THE PRODUCTION OF ETHANOL**

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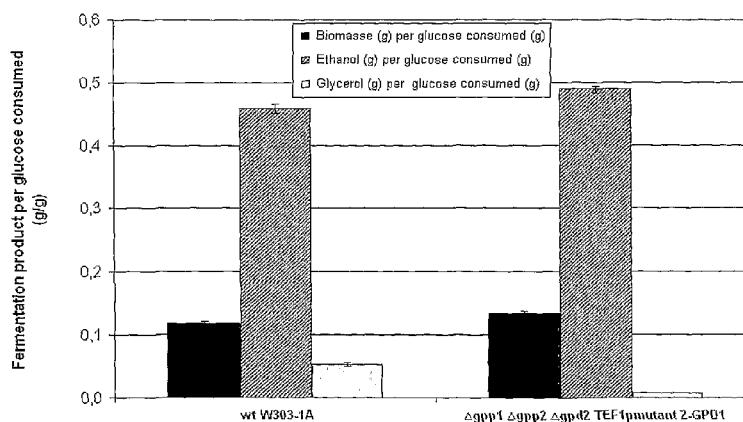
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(57) **ABSTRACT**

The invention relates to a method of modifying a yeast cell for the production of ethanol. According to some embodiments of the invention, the activity of the Gpd1 protein and/or the Gpd2 protein is reduced.

7 Claims, 6 Drawing Sheets



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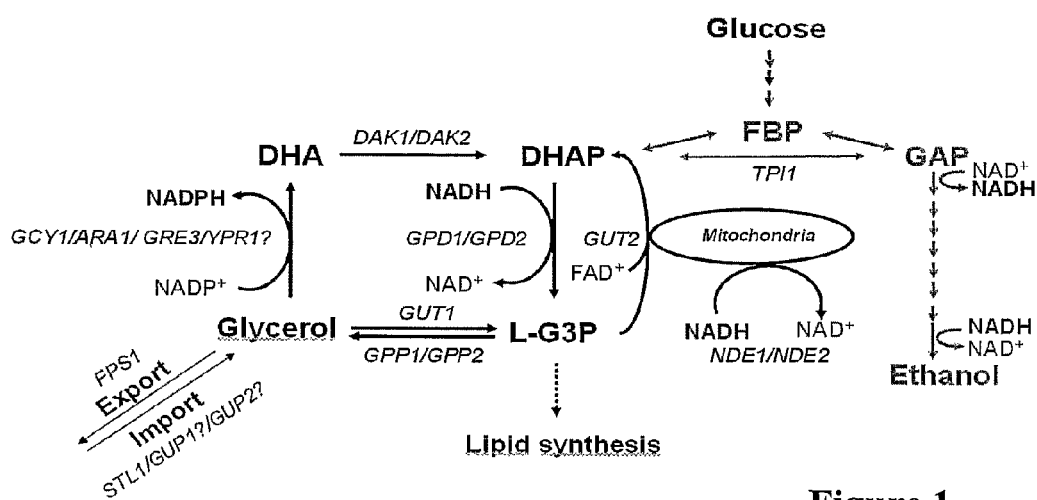


Figure 1

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<i>TEF1p</i> unmutated	1.00 + 0.00	ATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTCCTGGACTCCGGCATCGCCGTACCACTTCAAAAC
Mutant 2	0.07 + 0.01	ACGGCTCTAAAGTGGTTCGGCTCCCTTTTACTCTCCAGGTTTCTCTAGACATCCGGCATCGCCGTACCACTTCAAAAGC
<i>TEF1p</i> unmutated		ACCCAAGCACAGCATACTAAATTTCCCTCTTTCTTCTCTAGGGTGTCTTAATACCCGTAATAAGGTTTGGAAAAG
Mutant 2		CCCCAAGCGCAGCATACCAAAATCTCCCTCTTTCTCTCTAGGGTGTCTACTAGTTACTCGTACTAAAGGGTTGGGAAG
<i>TEF1p</i> unmutated		AAAAAGAGACCGCTCGTTCTTTTCTTCTCGTGGAAAAAGCAATAAAATTTTATCACGTTTCTTTTCTTGAAAAT
Mutant 2		GAGAAAGAGACCGCTCGTTCTTTCTTCTTCTCGTGGAAAAAGCAATAAGGTTTATCATGTCTCTTTCTCTTGAGAAC
<i>TEF1p</i> unmutated		TTTTTTTTTGAATTTTTTCTCTTTTCGATGACCTCCCATTTGATATTTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTTC
Mutant 2		CTTTTTCTTCGATCTTTGTCTCTCTTTTCGACGGCCCTCCCTGGTATTTAGTTAATGAACGGTCTTCAACCTCTCAAGTTTC
<i>TEF1p</i> unmutated		AGTTTCATTTTCTTTGTTCTATTACAACTTTTCTTACTTCTTTGCTCATTTAGAAAGAAAGCATAGCAATCTAATCTAAGTTT
Mutant 2		AGTTTCGTTTGTCCGGTCTATTACGACCGTTCTTACTTCTCTCAGTAGAAAGGGAGCATAGCAATCTAATCTCAAGTTT

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Figure 2

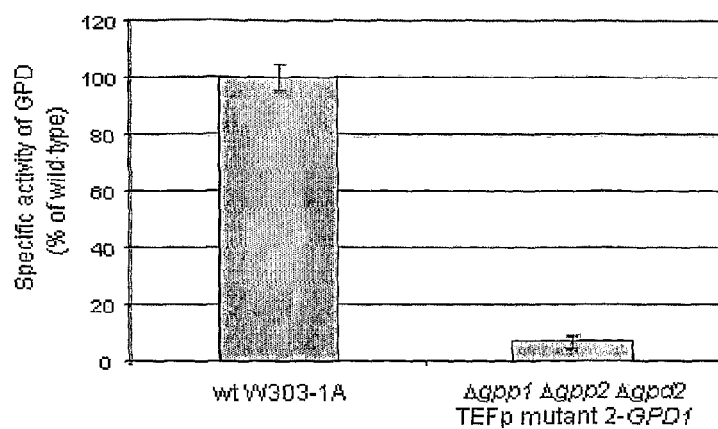


Figure 3

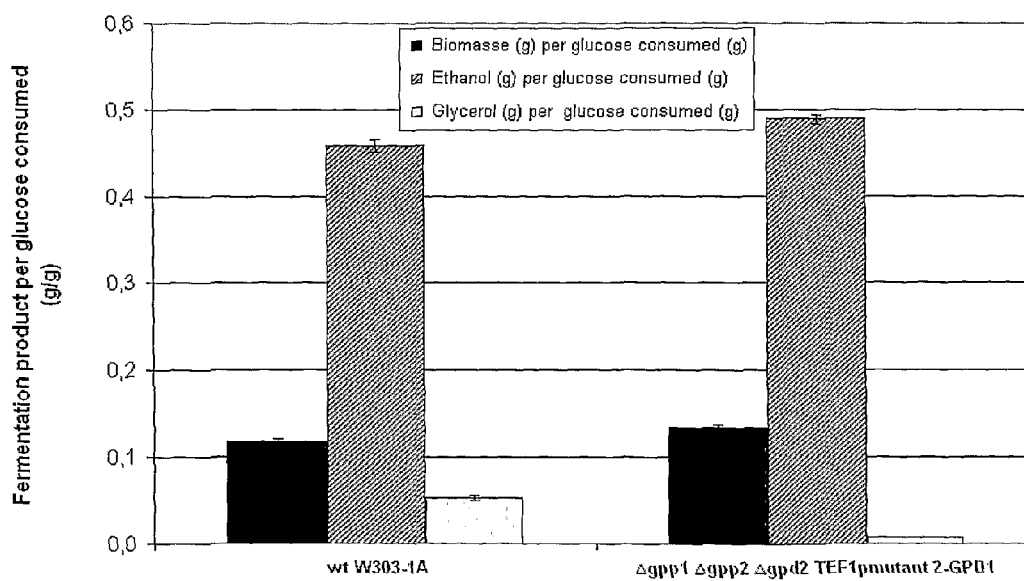
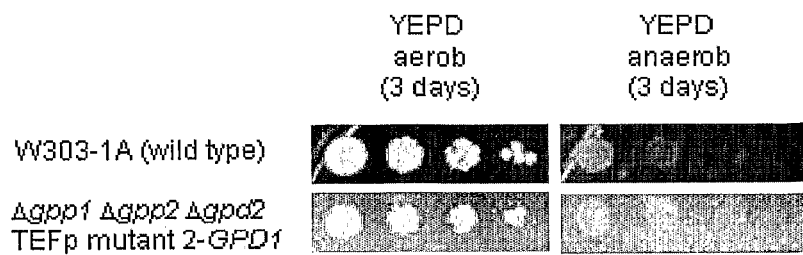
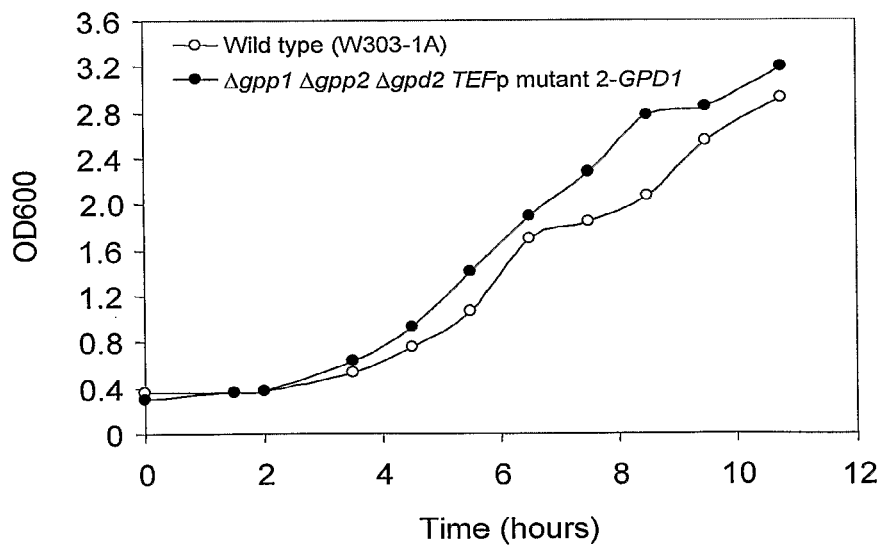


Figure 4

**Figure 5****Figure 6**

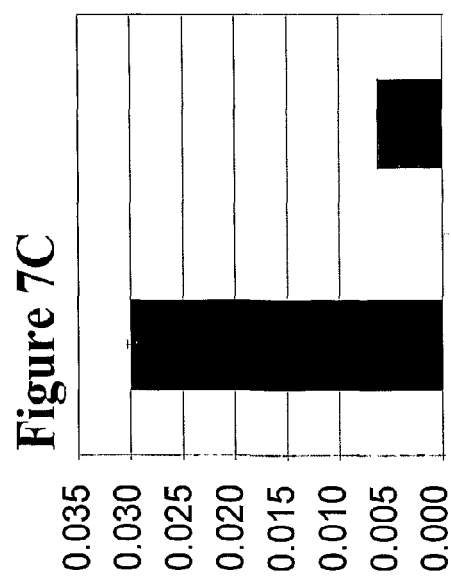
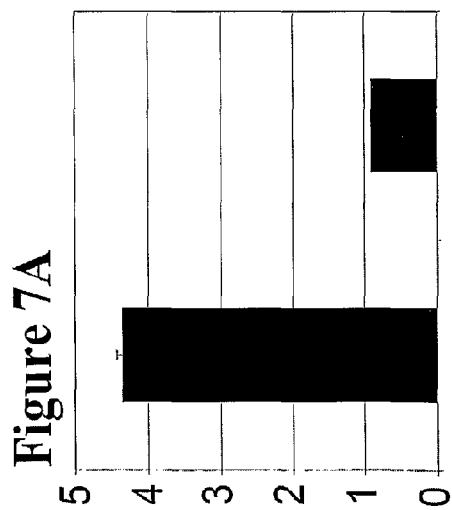
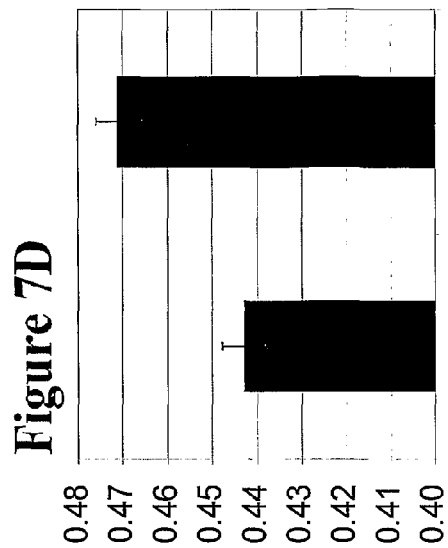
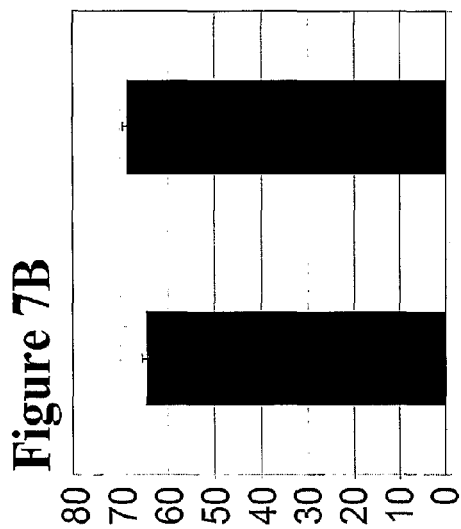


Figure 7F

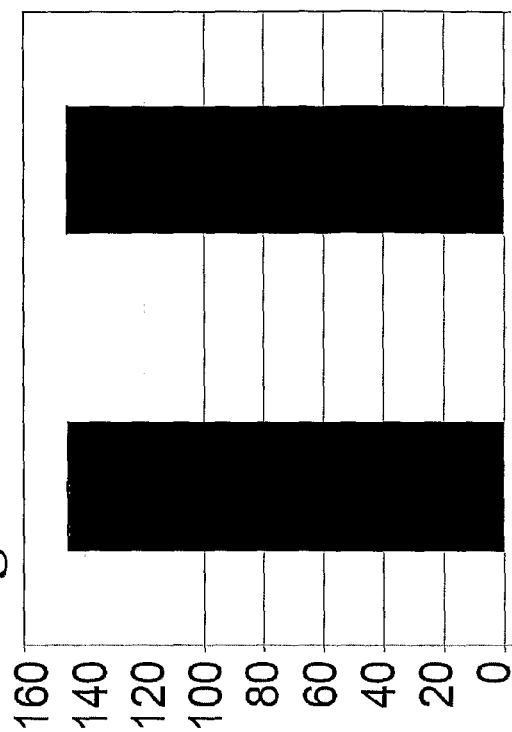
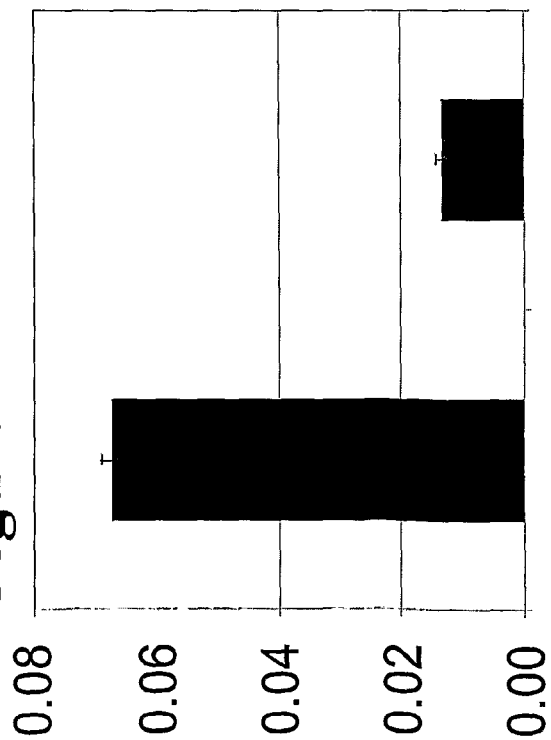


Figure 7E



METHOD OF MODIFYING A YEAST CELL FOR THE PRODUCTION OF ETHANOL

CROSS-REFERENCE TO RELATED APPLICATION

This application is a National Stage filing of International Application Serial No. PCT/IB2008/003672, filed Oct. 29, 2008, which claims priority to European Patent Application Serial No. 07 021 129.7 filed Oct. 29, 2007, the disclosures of which are expressly incorporated herein by reference.

The invention pertains to a method of modifying a yeast cell, in particular for the production of ethanol. The invention furthermore pertains to a method for producing ethanol from biomass.

Bio-ethanol is a promising alternative to fossil fuels. The increasing interest in renewable biofuels mainly results from the fact that world fossil fuels are limited. Moreover, there is the tendency to decrease the dependency of importing oil. The European Commission has planned to progressively substitute 20% of conventional fossil fuels by alternative fuels in the transport sector by 2020 (5.75% by 2010). One technical pathway is to produce bio-ethanol via microbial fermentation from various domestic crops (biomass).

Bio-ethanol production from sugar and starch containing biomass is common in Brazil and the United States. The yeast *Saccharomyces* (*S.*) *cerevisiae* has been traditionally used in this process. In fact, the yeast *S. cerevisiae* has outstanding properties for bio-ethanol production. In particular, its high tolerance to the conditions which occur during industrial ethanol production will hardly allow other microorganisms to displace yeast in this field.

Glycerol is formed by *S. cerevisiae* as a by-product during glucose catabolism beside the main fermentation products: ethanol, carbon dioxide and biomass. The carbon flux towards glycerol is quite substantial and can amount up to 0.1 g glycerol per gram glucose (Alfenore et al., 2004; Aldiguier et al., 2004).

Glycerol biosynthesis from the glycolytic intermediate dihydroxyacetone phosphate (DHAP) in *S. cerevisiae* is performed by two enzymatic steps catalyzed by the glycerol 3-phosphate dehydrogenase (GPDH) and the glycerol 3-phosphatase (GPP) (see also FIG. 1). Each enzyme is encoded by two isogenes GPD1/GPD2 and GPP1/GPP2, respectively.

Glycerol biosynthesis has essential roles in *S. cerevisiae*. One of the most important functions is maintaining cytosolic redox balance, especially under anaerobic conditions, and probably also under aerobic conditions when sugar concentration is high (Crabtree effect) (Ansell et al., 1997; Bakker et al., 2001; Rigoulet et al., 2004; Valadi et al., 2004). The glycerol biosynthetic pathway is also involved in the biosynthesis of glycerophospholipids and triacylglycerols which are formed from L-glycerol 3-phosphate (Kohlwein et al., 1996; Mullner and Daum, 2004). In addition, intracellular glycerol is involved in osmoadaptation (Hohmann, 2002), oxidative stress protection (Pahlman et al., 2001), and response to heat shock (Siderius et al., 2000). Responses to elevated temperatures and high osmolarity involve several signaling pathways including the protein kinase C pathway and the HOG pathway, which regulate intracellular levels of glycerol (Hohmann, 2002; Wojda et al., 2003).

In theory, the redirection of carbon flux in *S. cerevisiae* towards the ethanol synthetic pathway by eliminating glycerol formation could increase the ethanol yield by at least 10%. Moreover, reduction of glycerol in the fermentation broth would lead to a decrease in ethanol extraction costs as

glycerol has caused problems in the distillation units and separation processes after the fermentation stage. In addition, waste volumes would be reduced. Under practical aspects, however, reducing glycerol formation without negatively affecting the cells' fitness is extremely challenging due to the various biological functions of the glycerol biosynthetic pathway, as will now be outlined with reference to previous studies.

The first metabolic engineering approach to reduce glycerol was reported a few years ago (Nissen et al., 2000a). Ethanol yield in aerobic batch fermentations was increased by 12% when glycerol formation was completely abolished by deleting GPD1 and GPD2. Growth of this double mutant was severely affected even in the presence of oxygen. Therefore, the volumetric ethanol productivity obtained with this approach was far from industrial relevance. The fact that the growth of the *gpd1Δ gpd2Δ* double mutant was strongly impaired has been explained by a limited capacity of respiratory NADH reoxidation (by the external NADH dehydrogenases Nde1p, Nde2p and the mitochondrial L-G3P/DHAP shuttle) (Nissen et al., 2000a) which are the only pathways for reoxidizing excess cytosolic NADH when GPD is absent.

Other attempts to reduce glycerol formation relied on the introduction of bacterial transhydrogenases into yeast. These approaches failed since, on one hand, the *Azotobacter vinelandii* transhydrogenase produced the opposite expected effect (Nissen et al., 2001), and on the other hand, the membrane-bound transhydrogenase from *Escherichia coli* remained localized in the membrane of the endoplasmic reticulum (Anderlund et al., 1999).

A quite successful strategy to improve ethanol yield has been the metabolic engineering of the ammonium assimilation, reducing the NADH production during amino acid biosynthesis (Nissen et al., 2000b). The glycerol yield was reduced by 38% and the ethanol yield increased by 10%. However, for proper function, this approach requires that yeast utilizes ammonium as a source of nitrogen. Industrial media often contain amino acids, a fact which will considerably reduce the success of this approach under industrial relevant conditions.

Recently, an in silico study was carried out using a genome-scale *S. cerevisiae* metabolic model in order to evaluate possible metabolic engineering strategies to improve ethanol yield in *S. cerevisiae* (Bro et al., 2005). These approaches have been designed to prevent the production of excess NADH through biomass synthesis, and hence, reduce the need to produce glycerol. Based on authors' predictions, several approaches should be able to increase ethanol yield by up to 10.4%. One of the predicted strategies was tested in vivo, but in contrast to theory, only resulted in a 3% increase of ethanol yield.

Therefore, the metabolic engineering approaches mentioned above have no or only marginal impact on ethanol productivity under industrially relevant conditions due to the limitations in ethanol yield, growth or medium dependency. Moreover, it remains questionable if the current and predicted approaches would prove successful under high ethanol and thermal stress of industrial fermentations as they do not take into account the cells' need for intracellular glycerol.

DESCRIPTION OF THE INVENTION

The problem underlying the present invention therefore was to increase the conversion yield from fermentable biomass constituents into ethanol by yeast and, in effect, to

increase the economic efficiency of bio-ethanol plants. One way to solve this goal is to reduce the production of the by-product glycerol.

A particular challenge in solving this problem lies in the fact that the complete elimination of glycerol formation has proven to be unsuccessful, as the glycerol biosynthetic pathway has several important functions for cell growth and stress tolerance.

Instead, the inventors surprisingly found a strategy for modifying a wild type yeast cell that leads to an increased yield of ethanol from sugars, i.e. fermentable sugars present in hydrolysates of plant biomass, but at the same time does not have a negative influence on the growth rate of the yeast cells or the biomass yield. According to the invention, this is achieved by reducing (but not eliminating) the activity of the Gpd1 protein and/or the Gpd2 protein when compared to the activity of these proteins in a wild-type cell.

In effect, a higher ethanol yield, titer and specific productivity compared to the isogenic wild-type strain is achieved through the invention. Also, the metabolic pathway modification has the additional advantage to lower the costs for product recovery and reduces waste volumes.

The term "reducing the activity" is meant not to include the elimination of the activity of the protein. Moreover, the term "activity" refers to the in vivo metabolic flux through the particular protein, which, according to the definition of the term "reducing the activity" is not meant to include the complete blockage of this metabolic flux. Instead, the crux of the invention lies in the reduction of the activity of the Gpd1 protein and/or the Gpd2 protein, but at the same time providing a minimum activity of Gpd1 protein and/or the Gpd2 protein in order to allow for the production of substances downstream of these enzymes (such as glycerol) that are necessary to maintain a normal growth rate.

This result can be achieved in different ways: First, it is possible to reduce the activity of the Gpd1 protein and to eliminate the activity of the Gpd2 protein. Secondly, the activity of the Gpd1 protein can be eliminated and the activity of the Gpd2 protein can be reduced. Thirdly, the activity of both the Gpd1 protein and the Gpd2 protein can be reduced. Which of the three options leads to best results depending e.g. on the type of yeast strain used or the growth conditions can be determined by a person of skill in the art without undue burden.

The yeast cells according to the invention are useful for any application in which the production of glycerol in the cell needs to be minimized to a level that does not negatively influence the growth rate of the cell.

The reduction of the activity of the Gpd1 protein and/or the Gpd2 protein can be achieved in several ways that will now be outlined (under a) to e)). It lies within the inventive concept that one or a combination of the given possibilities can be used for a reduction in protein activity.

a) One way is to reduce the expression of the GPD1 gene and/or the GPD2 gene, which leads to a reduction of the protein in the cell.

This can be achieved in one embodiment of the invention by expressing the GPD1 gene and/or the GPD2 gene by a weak promoter that is operably linked to the GPD1 gene and/or operably linked to the GPD2 gene. A promoter is weak when the transcription rate of the gene is reduced to at least 20% or 15%, preferably to at least 10%, most preferably to at least 7% or 5% of the transcription rate of that gene expressed under the TEF1 wild type promoter (SEQ ID NO 11). Ways of measuring the strength of a promoter are known to a person of skill in the art, such as using a reporter gene like luciferase or

green fluorescent protein (GFP), measuring the mRNA levels, e.g. using Northern blot or real-time reverse transcriptase PCR; on the protein level by Western blotting; or through measurements of the specific enzyme activity.

It is preferred that the expression of the GPD1 gene and/or the GPD2 gene is reduced by at least 50%, at least 60%, or at least 70% compared to its expression in a wild type cell under its wild type promoter. It is of particular advantage to reduce expression by at least 80%, or at least 90%, and it is most preferred to reduce the expression by at least 95%, or at least 99%, compared to the expression of the particular gene in a wild type yeast cell, i.e. a yeast cell with a native promoter.

In a preferred embodiment, the weak promoter is a promoter according to SEQ ID NO 5 or 6. The promoter according to SEQ ID NO 5 leads to a transcription rate of 7% and the promoter according to SEQ ID NO 6 leads to a transcription rate of 16% of the transcription rate caused by the TEF1 wild type promoter (SEQ ID NO 11) (Nevoigt et al., 2006).

b) The reduction of the activity of the Gpd1 protein and/or the Gpd2 protein can also be achieved by providing or expressing an antisense molecule, such as an RNA molecule, to the GPD1 and/or the GPD2 mRNA to impede translation of the mRNA into a protein.

It is preferred that the antisense molecule has a sequence that hybridizes with the mRNA according to SEQ ID NO 1 or 2. In another embodiment, the antisense molecule hybridizes with or is reverse complementary to any 10 to 30 bases, preferably to any 18 to 23 bases of the mRNA according to SEQ ID NO 1 or 2.

When using antisense molecules, it is generally preferred to design them against untranslated regions of the mRNA.

Another possible means of reducing the activity of the Gpd1 protein and/or the Gpd2 protein are ribozymes, which can catalytically cleave the gpd1 and/or the gpd2 mRNA.

Several approaches have been developed based on antisense molecules and ribozymes to regulate gene expression, such as riboswitches. Riboswitches contain aptamer domain sites comprising highly specific pockets in the 5' untranslated region of the mRNAs that bind small molecules or ligands. Upon binding of a ligand to an aptamer site a conformational change in the RNA structure leads to a change in gene expression.

Moreover, it is possible to target transcription factors to lower the transcription rate of the Gpd1 protein and/or the Gpd2 protein. It has, e.g. been described that overexpression of Yig1p leads to a decreased activity of GPP (Granath et al, 2005).

c) Alternatively, the reduction of the activity of the Gpd1 protein and/or the Gpd2 protein can also be achieved by providing or expressing a functional antagonist to the Gpd1 and/or the Gpd2 protein, that functionally inhibits the enzymatic activity of the respective protein.

d) Also, the reduction of the activity of the Gpd1 protein and/or the Gpd2 protein can be achieved by providing or expressing a mutated form of the Gpd1 and/or the Gpd2 protein.

Such a mutant exhibits a functional inhibition of the enzymatic activity that can bear a mutation in a functional domain of the protein, such as the active center or a binding or recognition domain and leads to a reduced enzymatic activity of the respective protein without abolishing its function.

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e) Finally, the reduction of the activity of the Gpd1 protein and/or the Gpd2 protein can also be achieved by providing a small inhibitory molecule for inhibiting the Gpd1 and/or the Gpd2 protein.

The amino acid sequences of the Gpd1 protein, the Gpd2 protein, the Gpp1 protein, and the Gpp2 protein from *S. cerevisiae* can be found as SEQ ID NO 26, 27, 28, and 29, respectively. For other yeast species, a person of skill in the art can identify the respective amino acid sequence.

In a preferred embodiment of the method according to the invention, the Gpp1 protein and/or the Gpp2 protein, i.e. another key enzyme of the glycerol pathway, is also reduced in its activity in addition to the activity reduction of the Gpd1 protein and/or the Gpd2 protein.

It is possible either to reduce the activity of the Gpp1 and eliminate the activity of the Gpp2 protein, to eliminate the activity of the Gpp1 and reduce the activity of the Gpp2 protein, or to reduce both the activity of the Gpp1 and of the Gpp2 protein. In addition, it is also possible to eliminate both the activity of the Gpp1 and of the Gpp2 protein in one embodiment, as will be shown in the examples.

The means that can be used for reducing the activity of the Gpd1 protein and/or the Gpd2 protein are equivalent to the means explained above and apply in an equivalent fashion also for the activity reduction of Gpp1 and/or Gpp2, as will be realized by a person of skill in the art. Accordingly, the GPP activity can be reduced by reducing the expression of the Gpp1 and/or the Gpp2 protein, by providing an antisense molecule to the GPP1 (SEQ ID NO 3) and/or the GPP2 mRNA (SEQ ID NO 4), by providing an antagonist to the Gpp1 and/or the Gpp2 protein, by providing a mutated form of the Gpp1 protein and/or the Gpp2 protein or by providing a small inhibitory molecule such as fluoride, which has been described as an unspecific inhibitor of phosphatases, for inhibiting the Gpp1 protein and/or the Gpp2 protein. For details regarding these reduction means, reference is made to the description given above.

The present invention can generally be used with any yeast strain, such as *S. cerevisiae* and closely related species (i.e. other species of the genus *Saccharomyces*). Other Non-*Saccharomyces* yeast species, especially those which show ethanolic fermentation and have the ability to ferment pentoses such as *Pichia* (P.) *stipitis*, are also preferred. It is particularly preferred to use strains that are advantageous in industrial applications, such as the prototrophic *S. cerevisiae* yeast strain CEN.PK113-7D. Other suitable strains are known to a person of skill in the art.

It will be understood by a person of skill in the art that when using a diploid or polyploid strain, it becomes necessary to reduce the activities of the Gpp1p and/or Gpp2p as well as possibly Gpd1p and/or Gpd2p in all of the alleles present in order to achieve the necessary reduction in protein activity.

The underlying problem is also solved by a yeast cell, in particular a genetically modified yeast cell that is obtainable through a method as described above.

Specifically, in such a yeast cell, the activity of the Gpd1p and/or Gpd2p protein is reduced in comparison to the activity of said proteins in a wild-type yeast cell, i.e. in a yeast cell with a normal protein activity (normal flux) and a normal growth rate, or, put differently, in comparison to a yeast cell in which the modifications present in the genetically modified yeast cell according to the invention that lead to the reduced activity of the Gpd1 and/or Gpd2 protein are not present.

For the preferred amount of reduction of protein activity, reference is made to the description above.

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Means for reducing the activity of said proteins were described above, the application of which leads to a yeast cell in which

the expression of the GPD1 gene and/or gpd2 gene is reduced,

an antisense molecule to the GPD1 and/or gpd2 mRNA, e.g. in the form of an RNA molecule, is present,

a functional antagonist to the Gpd1 protein and/or Gpd2 protein is present,

a mutated form of a Gpd1 protein and/or Gpd2 protein, that is functionally inhibited is present, and/or

a small inhibitory molecule for inhibiting the Gpd1 and/or the Gpd2 protein is present.

Further characteristics of such a yeast cell according to the invention were described above in relation to the method according to the invention.

The underlying problem is also solved through the use of a yeast cell, in particular a genetically modified yeast cell as describe above for producing ethanol from biomass. This can be achieved by providing a modified yeast cell as described above, providing biomass and growing the yeast cell in the presence of the biomass, as well as obtaining the ethanol. In general, the yeast cells according to the invention can be used in any application in which high glycerol production in the cell is to be avoided, since the reduction of glycerol according to the method described here does not lead to smaller growth rates.

The term biomass when used together with a method of producing ethanol, is meant to refer to plant and plant-derived materials, such as starch, sugar, cellulose, hemicellulose, in particular from sugar cane, sugar beet, corn, grain, etc.

The underlying problem is furthermore solved by a method for the production of ethanol which comprises the following steps:

providing a yeast cell as described above,

providing biomass, and

growing the yeast cell in the presence of the biomass under conditions that allow for the production of ethanol.

As will be evident to a person of skill in the art, it might be necessary or advantageous to treat the biomass chemically, enzymatically or mechanically prior to growing the yeast together with the biomass in order to facilitate fermentation. Methods for such treatments are known to a person of skill in the art.

As shown by Alfenore et al., 2004, the production of glycerol can also be reduced by adapting the growth conditions of the yeast cell. Particularly the aeration conditions and the composition of the medium can have a large influence on glycerol production and therefore on ethanol production.

FIGURES

FIG. 1

The pathways involved in glycerol metabolism in *Saccharomyces cerevisiae* are shown. Glycerol is formed from glycolytic dihydroxyacetone phosphate (DHAP) by the action of both glycerol-3-phosphate dehydrogenase (GPD encoded by GPD1 and GPD2) and glycerol-3-phosphatase (G3Pase encoded by GPP1 and GPP2). Gut1p and Gut2p are responsible for the utilization of glycerol. The pathways for biosynthesis and metabolism of glycerol in *S. cerevisiae* have been reviewed by Nevoigt and Stahl (1997). The Fps1p channel is the mediator of the major part of glycerol passive diffusion (Oliveira et al., 2003). Yeast cells take in glycerol via transporter Stt1p and probably also via Gup1p and Gup2p (Ferreira et al., 2005). Glycerol is converted to dihydroxyacetone (DHA) by NADP+-dependent glycerol dehydrogenase

(GDH). The genes ARA1, GCY1, GRE3, YPR1 are suggested to contribute to this activity (Izawa et al., 2004); however, others reported that no activity of this enzyme at all is detectable, a result which has put the relevance of the DHA pathway for *S. cerevisiae* into question (Norbeck and Blomberg, 1997). DAK1 and DAK2 encode dihydroxyacetone kinase (Molin et al., 2003). NDE1 and NDE2 encode the external NADH dehydrogenase in yeast which is able to directly reoxidize cytosolic NADH transferring the electrons to the respiratory chain. DHAP: dihydroxyacetone phosphate, GAP: glyceraldehyde 3-phosphate, L-G3P: L-glycerol 3-phosphate, DHA: dihydroxyacetone, FBP: 1,6-fructose biphosphate, and the TPI1: gene encoding triose phosphate isomerase

FIG. 2

FIG. 2 shows the sequence alignment of the unmutated TEF1 promoter of *Saccharomyces cerevisiae* and TEF1 promoter mutant 2. The normalized promoter strength is shown.

FIG. 3

Specific activity of glycerol 3-phosphate dehydrogenase of the engineered *Saccharomyces cerevisiae* strain related to the isogenic wild type. 1 Unit is defined as the conversion of 1 μ mole of substrate per minute and mg protein.

FIG. 4

Ethanol, biomass and glycerol yields in gram per glucose consumed of the engineered *Saccharomyces cerevisiae* strain and the isogenic wild type after depletion of glucose in fermentations of YEPD medium under oxygen-limited conditions.

FIG. 5

The result of a growth experiment of the engineered *Saccharomyces cerevisiae* strain and the isogenic wild type on YEPD medium (agar-plates) under aerobic and anaerobic conditions is shown.

FIG. 6

The result of a growth experiment of the engineered *Saccharomyces cerevisiae* strain and the isogenic wild type in liquid YEPD medium under aerobic conditions is shown.

FIG. 7

Ethanol, glycerol production and sugar consumption after batch fermentation under oxygen-limited conditions at 30° C. in wheat mash. Wheat mash was completely saccharified and centrifuged before fermentation was started. The hydrolysate contained roughly 143 g/l total sugar, i.e. glucose and fructose. Oxygen-limited conditions were obtained by closing the Erlenmeyer flasks with air-locks which allowed the release of gases. Mixing was carried out using a magnetic stirrer set at 200 rpm. For this experiment, the prototrophic *S. cerevisiae* yeast strain CEN.PK113-7D and a derivative deleted in GPD2 and carrying modifications of the GPD1 promoter (a TEF1 promoter mutant version (mutant promoter 2, SEQ ID NO 5) and the loxP-KmR-loxP sequence as a selectable marker) was used. The genes encoding GPP1 and GPP2 were not modified in this strain.

In all panels A to F of FIG. 7, the left bar shows: CEN.PK113-7D, 100% GPD activity, and the right bar shows CEN.PK113-7D, 6% GPD activity.

The y-axis of the panels is as follows:

7A	Final glycerol concentration (g/l)
7B	Final ethanol concentration (g/l)
7C	Glycerol yield (g/g glucose, fructose consumed)
7D	Ethanol yield (g/g glucose, fructose consumed)
7E	Ration: glycerol/ethanol (g/g)
7F	Sugar (glucose, fructose) consumed (g/l)

EXAMPLES

Material and Methods

Media:

YEPD medium (1% yeast extract, 2% peptone, 2% glucose)

Yeast Strains:

The yeast strains generated in this study originate from *S. cerevisiae* laboratory strain W303-1A (Table 1). The strain YA103 corresponding to a *gpp1Δ gpp2Δ* double deletion strain has been published by Pahlman et al. (2001).

Further Genetic Modifications of *S. cerevisiae* Strain YA103:

1. Deletion of GPD2 Gene/Abolishment of GPD2 Expression

The GPD2 gene was disrupted in the strain YA103 by the method described by (Guldener et al., 1996) using pUG72 (Guldener et al., 2002) as a template and the primers P29 and P30 (Table 2). Disruption of GPD2 was checked by diagnostic PCR using the primer pair P33/P34 (Table 2). Selection of positive transformants was carried out on agar plates containing CSM-medium lacking uracil. The resulting strain has been referred as to EN-GGG (Table 1).

2. Down-Regulation of GPD1 Expression

The native chromosomal GPD1 promoter in the strain EN-GGG was replaced by the promoter replacement cassette amplified from genomic DNA of a yeast strain derived from laboratory yeast BY4741 bearing the mutated TEF promoter with the lowest activity (Nevoigt et al., 2006) in place of the native GPD1 promoter. The primers P9 (SEQ ID NO 7) and P10 (SEQ ID NO 8) were used for PCR amplification of the promoter replacement cassette (including the loxP-K.I.LEU2-loxP sequence as a selectable marker). PCR conditions were as previously published (Nevoigt et al., 2006). Two 100 μ l PCR aliquots were combined, precipitated used for transformation as described by Guldener et al. (1996). Selection of positive transformants was carried out on agar plates containing CSM-medium lacking leucine.

Correct integration of the promoter replacement cassette was checked by diagnostic PCR using primer combination P9 (SEQ ID NO 7)/P12 (SEQ ID NO 10) and P11 (SEQ ID NO 9)/P12 (SEQ ID NO 10) (Table 2). The resulting strain has been referred as to EN-G46a (Table 1).

TABLE 1

<i>S. cerevisiae</i> strains used in the examples		
Strain	Genotype	Reference
W303-1A*	MATa	Thomas and Roth-
YA103*	MATa <i>gpp1Δ::kanMX4 gpp2Δ::HIS3</i>	stein (1989)
EN-GGG*	MATa <i>gpp1Δ::kanMX4 gpp2Δ::HIS3</i>	Pahlman et al.
	<i>gpd2Δ::K.I.URA3</i>	(2001)
EN-G46a*	MATa <i>gpp1Δ::kanMX4 gpp2Δ::HIS3</i>	
	<i>gpd2Δ::K.I.URA3</i>	
	<i>gpd1p::TEFmut2::K.I.LEU2</i>	

*These strains harbor additional mutations as follows: *leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*

TABLE 2

Primers used		
Use/name	Sequence	SEQ ID NO
Amplification of the promoter replacement cassette including the TEF1 promoter mutant version (mutant promoter 2 described in Nevoigt et al., 2006) and the loxP-K.1.LEU2-loxP sequence as a selectable marker:		
P9 (binds upstream GPD1 prom.)	cccaaggcaggacagttacc	SEQ ID NO 7
P10 (binds in GPD1 cod. seq.)	agcaccagatagagcaccaca	SEQ ID NO 8
Diagnostic PCR to check the correct integration of the promoter replacement cassette:		
P11 (binds in K.1.LEU2)	ggaccaccaacagcacctagt	SEQ ID NO 9
P12 (binds downstream integration site in GPD1 coding sequence)	gtaagcaactgttgtttcaga	SEQ ID NO 10
Deletion of GPD2 using loxP-K.1.URA3-loxP as a selectable marker:		
P29	atgcttgctgtgcagaagattaacaagatacacattcctt	SEQ ID NO 12
P30	agatcccaataacaacagatcacg	SEQ ID NO 13
	cgatgtctagctcttcaatcatctccggtaggctttcca	
Diagnostic PCR to check the disruption of GPD2:		
P33	ggtagattcaattctctttccc	SEQ ID NO 14
P34	aggcaacaggaaagatcagagg	SEQ ID NO 15

Oxygen-Limited Batch-Fermentations and Determination of Product Yields

1st Day:

1st pre-culture: inoculate 20 ml YEPD with 500 µl glycerol stock

Incubate over night at 30° C. at a shaker (170 rpm) for 20 hours

2nd Day:

2nd preculture: inoculate 150 ml YEPD with 1.5 ml of the first preculture

Incubate over night at 30° C. at a shaker (170 rpm) for 20 hours

3rd Day:

Centrifuge 2nd preculture (10 min, 5000 rpm, 4° C.) and wash the cells once with distilled water

Inoculation of main culture: inoculate 100 ml YEPD in 100 ml—Schott flasks by adjusting an OD of 0.2

Immediately, samples were taken for determination of initial concentrations of glycerol, ethanol, glucose and biomass

Add a magnetic stirrer and close the flasks with air locks to ensure release of gases but prevent oxygen intake

Stir the culture for 24 h at 28° C. and 300 rpm

4th Day:

Samples (2×1 ml) were taken, centrifuged (10 min, 12000 rpm, 4° C.) and the supernatants were stored at -20° C. until glycerol, ethanol and glucose concentrations were measured. The measurements of glucose and fermentation products were carried out as previously described (Nevoigt and Stahl, 1996). Yeast dry weight (biomass) at the end of fermentation was determined by filtering 30 ml of the culture using pre-weighted nitrocellulose filters (pore size 0.45 µm). The filters with the cells were washed with distilled water and dried until the weight reached a stable value.

Determination of Specific Activity of Glycerol 3-Phosphate Dehydrogenase

In vitro enzyme activities were, in general, determined during logarithmic growth, i.e. when cell density was about 1 during the batch fermentations. Yeast cells were broken by

vortexing with glass beads (0.5 mm in diameter) for 15 min at 4° C. in accordance with a previously described method (Ciriacy, 1975). In order to assay GPD, approximately 3×10⁹ cells were harvested and homogenized in 3 ml triethanolamine buffer (Blomberg and Adler, 1989; Andre et al., 1991) containing 0.2 mmol/l-phenylmethyl-sulphonylfluoride and 2 g glass beads. The homogenate was centrifuged in each case at 12 000 g and 4° C. for 15 min. The supernatant was used after desalting by passage through a Sephadex G-25 column. (Pharmacia PD-10, Pharmacia Fine Chemicals, Sweden). GPD was assayed in imidazole buffer at pH 7.0 in accordance to Gancedo et al. (1968). Protein concentration was measured by the Coomassie blue method (Bradford, 1976), using bovine serum albumin A 3350 (Sigma Chemical Co., St Louis, Mo.) as a standard (Nevoigt and Stahl, 1996). Growth on Agar Plates Under Aerobic and Anaerobic Conditions

Stationary phase cultures of the two strains in YEPD medium were diluted (decadal dilutions) and an aliquot was transferred to YEPD agar plates using a stamp. Plates were incubated for 3 days. Oxygen-free conditions were obtained by applying Anaerocult A (MERCK) in an airtight incubator.

Deletions of GPP1 and GPP2

Deletion of the GPP1 gene can be accomplished by the long flanking homology PCRtargeting technique (Pahlman et al, 2001). In the first step, a set of primers (TGTGTGAGT-TCCTCTTTTCTT (SEQ ID NO 16) and TCAAAGGCAT-TGCGATGGTT (SEQ ID NO 17)) was used to amplify a 263 base pair (bp) long portion of genomic DNA from *S. cerevisiae* W303, upstream from the third codon in the GPP1 ORF. A second set (CGCTAAGGATGACTTGTGTA (SEQ ID NO 18) and CTCTAACTTCTCGTCGTACT (SEQ ID NO 19)) was used to amplify a 358 bp fragment from the ninth codon in the GPP1 ORF upstream the stop codon. The 59-end of the primers adjacent to the insertion site carried 25 nucleotide extensions homologous to the 59 and 39 regions of the his-GMX6 or kanMX4 disruption cassette of plasmid pFA6a-hisGMX6 and pFA6-kanMX4. In the second PCR reaction, pFA6a-hisGMX6 and pFA6-kanMX4 were used as templates and the 59 and 39 homologous regions of the first PCR reac-

tion were fused to the disruption cassette by serving as primers together with the upstream forward and downstream reverse primers of the flanking regions, thus producing the ORF targeting cassette. This cassette was transformed into a haploid *S. cerevisiae* W303 strain, and independent transformants were selected for verification of GPP1 replacement. Using a set of primers (forward: CAAGCAGGAAATCCG-TATCA (SEQ ID NO 20) and reverse TCATATGGAG-CAATCCCACT (SEQ ID NO 21)) hybridizing upstream and downstream, respectively, of the disruption cassette chromosomal DNA was amplified. The length of the PCR products was verified by agarose-gel electrophoresis. The GPP2 ORF was disrupted in a similar way using a set of primers (CAAGTGAGGACTTTTCGGAT (SEQ ID NO 22) and GTAGTCAATCCCATTCGGAA (SEQ ID NO 23)) to amplify a 346-bp fragment upstream from the fourth codon in the ORF. The second set (GGACGATCTGTTGAAATGGT (SEQ ID NO 24) and CCTGTCCACTTTCAAGTTGCT (SEQ ID NO 25)) was used to amplify a 287-bp fragment from the seventh codon in the GPP2 ORF downstream the stop codon. Correct integration of the disruption modules into the GPP1 and GPP2 alleles was verified by PCR using appropriate primers.

Based on this strain, further deletions were introduced as described herein.

Preliminary Experiments

In initial studies, the inventors tested strains deleted in GPP for their ability to prevent glycerol formation in fuel bio-ethanol production. The complete elimination of GPD, a key enzyme in glycerol biosynthesis, was not straightforward. The main advantages of abolishing GPP activity, instead of GPD, have been seen in i) keeping the NADH reoxidizing step of glycerol biosynthesis (fulfilled by gene products of GPD1/2), and ii) providing L-G3P for anabolic purposes (FIG. 1).

Both single deletion strains (gpp1Δ and gpp2Δ) and a double deletion strain (gpp1Δgpp2Δ) of the laboratory yeast strain W301-1A were studied. The phenotypes of the different strains were characterized during dynamic ethanol fermentation processes in a highly instrumented bio-reactor in mineral medium under aerobic conditions. Comparative analysis of the wild-type strain and the different mutant strains led to the following conclusions:

- a single deletion of one of the two GPP genes did not lead to important phenotypic changes (growth, ethanol and glycerol production)

- the glycerol concentration was only decreased by 65% in the double deletion mutant gpp1Δ gpp2Δ but not abolished

- the gpp1Δ gpp2Δ double mutant showed a negatively affected growth rate (decreased by 65%) and a lower ethanol tolerance

The pathway of glycerol formation in a gpp1Δ gpp2Δ mutant is unknown. Moreover, the reasons for negatively affected growth and the lower ethanol tolerance in the double deletion mutant gpp1Δ gpp2Δ remain unclear. Nevertheless, data shows that complete deletion of GPP is also not straightforward to strongly improve ethanol productivity. GPP likely has another unknown but important function in the cell.

Our experiments show that growth of a gpp1Δ gpp2Δ mutant can be recovered to wild-type level after reducing GPD activity in this strain. It is therefore assumed, without wanting to be bound to theory, that a high intracellular accumulation of L-glycerol 3-phosphate is responsible for the growth defect of a gpp1Δ gpp2Δ mutant. This high level is reduced when GPD activity is reduced in the cell.

Hence, the inventors surprisingly found that cell fitness is maintained (in GPP wild-type) or restored (in cells with abolished GPP activity) if the activity of GPD, a key enzyme in the glycerol biosynthetic pathway is not completely abolished, but instead a minimal flux through the key enzyme required by the cell is maintained. This is in contrast to complete abolishment of GPD or GPP, as both proved to be detrimental for cell fitness.

Generation of Promoters of Graded Activities for Fine-Tuning Enzyme Activities

It is of crucial importance to have tools for fine-tuning enzyme activities in order to determine cells' minimal requirements with regard to the flux through the glycerol biosynthetic pathway. Recently, a robust and well-characterized collection of yeast promoter mutants of finely graded strengths was developed (Alper et al., 2005; Nevoigt et al., 2006). Using these promoter mutants, promoter replacement cassettes were created, which are now available in combination with two different genetically selectable markers. To show the utility of these promoter cassettes, they have been used to tune GPD1 expression in *S. cerevisiae* and analyze the impact on glycerol formation and biomass yield (Nevoigt et al., 2006).

Results

A *S. cerevisiae* laboratory strain was generated which carries deletions in the genes GPP1, GPP2 and GPD2 and which has a very low expression of GPD1 due to the fact that the native GPD1 promoter in the yeast genome was replaced by a weak promoter. This weak promoter (SEQ ID NO 5) was obtained from the TEF1 promoter mutant collection (TEFp mutant 2) created by Nevoigt et al. (2007) and is shown together with the TEF1 wild type promoter (SEQ ID NO 11) in FIG. 2.

This strain referred to as gpp1Δgpp2Δgpd2Δ TEF1pmut2-GPD1 (EN-G46a; Table 1) showed a GPD activity which was about 7% that of the isogenic wild type (FIG. 3). The gpp1Δgpp2Δgpd2Δ TEF1pmut2-GPD1 and the corresponding wild type were used to ferment 2% glucose in a complex medium (YEED) under oxygen limiting conditions (see Methods above). The engineered strain showed a glycerol yield per gram glucose consumed which was only 14.5% that of the wild type (FIG. 4). The ethanol yield (gram ethanol per gram glucose consumed) was 6.7% higher than the wild type yield (FIG. 4). Surprisingly, the final biomass yield (FIG. 4) was not influenced by the engineering of the glycerol pathway even though the conditions during the batch fermentation were quasi anaerobic (100 ml culture in 100 ml flasks closed with air-locks). The growth of both strains under aerobic and anaerobic conditions was also investigated using YEED agar plates and there was virtually no difference (FIG. 5). The growth in liquid YEED medium under aerobic conditions was also shown to be the same (FIG. 6). Both strains showed an average growth rate of 0.27 h⁻¹ during exponential growth phase.

Preliminary experiments have shown that the same result can be obtained by down-regulating GPD activity alone, i.e. without GPP1 and GPP2 deletions. Therefore, it seems that the deletions of GPP1 and GPP2 are not necessary for the invention.

INDUSTRIAL RELEVANCE OF THE RESULTS

The results obtained have a great impact on bio-ethanol production (including biofuels of the first generation) as more ethanol can be produced from the same amount of substrate (carbohydrates such as hydrolysates of starch, cellulose or hemicellulose). Moreover, glycerol production is strongly

reduced. This is also important because glycerol participates to the fouling of the distillation units in bio-ethanol production process.

This is the first time that glycerol production was strongly reduced without negatively influencing growth under oxygen limiting conditions. The increase in ethanol productivity is higher than described in the prior art due to the normal growth rate of the cells together with an increased production of ethanol at the expense of glycerol formation.

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cccgggcacc caaagtcccc acccacacca ccaatacgtg aacgggggcgc cccctgcagg      180
ccctctgcg cgcggcctcc gcgcttgctt ctctccctt ccttttcttt ttccagtttt      240
ccctattttg tccctttttc cgcacaacaa gtatcagaat ggggttcacg aatctatcca      300
acctaattcg cactagact ggcttggtat tggcagtttc gtagttatat atatactacc      360
atgagtgaag ctgttacgtt accttaaatt cttctccct ttaattttct ttatctttac      420
tctctacat aagacatcaa gaaacaattg tatattgtac accccccccc tccacaaaca      480
caaataattg taatataaag atgtctgctg ctgctgatag attaaactta acttccggcc      540
acttgaatgc tggtagaaag agaagttcct cttctgtttc tttgaaggct gccgaaaagc      600
ctttcaaggt tactgtgatt ggatctggtg actggggtag tactattgco aaggtggttg      660
ccgaaaattg taaggggatac ccagaagttt tcgctccaat agtacaaatg tgggtgttcg      720
aagaagagat caatggtgaa aaattgactg aatcataaaa tactagacat caaaacgtga      780
aatacttgcc tggcatcact ctaccgcaga atttggttgc taatccagac ttgattgatt      840
cagtcaagga tgtcgacatc atcgttttca acattccaca tcaatttttg ccccgatatc      900
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ttcaatgtgg tgctctatct ggtgctaaca ttgccaccga agtcgctcaa gaacactggt     1080
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accataaggt tctaaaggcc ttgttcacac gaccttactt ccacgttagt gtcacgaag	1200
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tcgtcgaagg tctaggctgg ggtaacaacg cttctgctgc catccaaaga gtcggtttgg	1320
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aagagtctgc tgggtgtgct gatttgatca ccacctgcgc tgggtgtaga aacgtcaagg	1440
ttgctaggct aatggctact tctggttaagg acgctggga atgtgaaaag gagttgttga	1500
atggccaatc cgctcaagg ttaattacct gcaaagaagt tcacgaatgg ttggaaacat	1560
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ttattattaa attaatacaa aatttagtaa ccaataaat ataaataaat atgtatattt	1860
aaattttaaa aaaaaatcc tatagagcaa aaggattttc cattataata ttagctgtac	1920
acctcttcg cattttttga ggggtgttac aacaccactc attcagaggc tgcggcaca	1980
gttgcttcta gcactctggc tcgctatgta tgggtgtatt ttaaataata aacaaagtgc	2040
cacaccttca ccaattatgt ctttaagaaa tggacaagtt ccaaagagct tgcccaaggc	2100
tcgacaagga tgtactttgg aatatctata ttcaagtacg tggcgcgcat atgtttgagt	2160
gtgcacacaa taaagg	2176

<210> SEQ ID NO 2

<211> LENGTH: 2323

<212> TYPE: DNA

<213> ORGANISM: *Saccharomyces cerevisiae*

<400> SEQUENCE: 2

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acgacgatgg ctctgccatt gttatattac gcttttgccg cgagggtgcc atgggttgct	180
gaggggaaga gtgttttagc tacggacctt ttgccattgt tattccgatt aatctattgt	240
tcagcagctc ttctctaccc tgcattctc gtattttttt tttttttttt tggttttact	300
tttttttctt cttgcctttt tttcttgta ctttttttct agtttttttt ccttccacta	360
agcttttttc ttgatttatc cttgggttct tctttctact ccttttagatt ttttttttat	420
atattaattt ttaagtttat gtatttttgt agattcaatt ctctttccct ttctttttcc	480
ttcgctcccc ttccttatca atgcttgctg tcagaagatt aacaagatac acattcctta	540
agcgaacgca tccggtgtta tatactcgtc gtgcatataa aattttgcct tcaagatcta	600
ctttcctaag aagatcatta ttacaaacac aactgcactc aaagatgact gctcacta	660
atatcaaaac gcacaaacac tgcatgagg accatcctat cagaagatcg gactctgccg	720
tgtcaattgt acatttgaaa cgtgcgccct tcaagggtac agtgattggt tctggttaact	780
ggggggaccac catcgccaaa gtcattgcgg aaaacacaga attgcattcc catatcttcg	840
agccagaggt gagaatgtgg gtttttgatg aaaagatcgg cgacgaaaat ctgacggata	900
tcataaatat aagacaccag aacgttaaat atctacccaa tattgacctg ccccataatc	960
tagtggccga tcctgatctt ttactctca tcaagggtgc tgacatcctt gttttcaaca	1020
tcctcatca atttttacca aacatagtc aacaattgca aggccacgtg gccctcatg	1080

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taagggccat ctcgtgtcta aaagggttcg agttgggctc caaggggtgtg caattgctat	1140
cctcctatgt tactgatgag ttaggaatcc aatgtggcgc actatctggt gcaaacttgg	1200
caccggaagt ggccaaggag catttggtccg aaaccacgt ggcttaccaa ctaccaagg	1260
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cttacttcca cgtcaatgtc atcgatgatg ttgctgggat atccattgcc ggtgccttga	1380
agaacgtcgt ggccacttga tgtggtttcg tagaaggat gggtggggg aacaatgcct	1440
ccgcagccat tcaaaggctg ggtttagggt aaattatcaa gttcggtaga atgtttttcc	1500
cagaatccaa agtcgagacc tactatcaag aatccgctgg tgttgcatg ctgatcacca	1560
cctgctcagg cggtagaaac gtcaagggtg ccacatacat ggccaagacc ggtaagtcat	1620
ccttggaagc agaaaaggaa ttgcttaacg gtcaatccgc ccaagggata atcacatgca	1680
gagaagtcca cgagtggcta caaacatgtg agttgaccca agaattccca ttattcgagg	1740
cagtctacca gatagtctac aacaacgtcc gcatggaaga cctaccggag atgattgaag	1800
agctagacat cgatgacgaa tagacactct cccccccct cccctctga tctttcctgt	1860
tgcctctttt tcccccaacc aatttatcat tatacacaag ttctacaact actactagta	1920
acattactac agttattata attttctatt ctctttttct ttaagaatct atcattaacg	1980
ttaatttcta tatatacata actaccatta tacacgtat tatcgtttac atatcacatc	2040
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tttctgttat ctatagccct taaagctgtt tcttcgagct ttttcactgc agtaattctc	2160
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tcatttatgt gatttttttag tgactcatgt cgcatttggc ccgttttttt ccgctgtcgc	2280
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<210> SEQ ID NO 3

<211> LENGTH: 1753

<212> TYPE: DNA

<213> ORGANISM: *Saccharomyces cerevisiae*

<400> SEQUENCE: 3

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gcgatcatca tcattaacat aattttttta acgctgtttg atgatggat gtgcttttat	180
tgttccttac tcaccttttc ctttgtgtct tttaattttg accattttga ccattttgac	240
ctttgatgat gtgtgagttc ctcttttctt tttttctttt ctttttctct tttttttct	300
tttcttactc tgttaatcac tttctttcct ttttgttc attgctgtct tgttcatttt	360
cgttcaattg ataatgtata taaatcttgc gtaagtatct cttgattgcc attttttct	420
ttccaagttt ccttggtatg aaacgtttca atgttttaaa atatatcaga acaacaaaag	480
caaatataca aaccatcgca atgcctttga ccacaaaacc tttatctttg aaaatcaacg	540
ccgctctatt cgatgttgac ggtaccatca tcactctcca accagccatt gctgctttct	600
ggagagattt cggtaaagac aagccttact tcgatgccga acacgttatt cacatctctc	660
acggttggag aacttacgat gccattgcca agttcgctcc agactttgct gatgaagaat	720
acgttaacaa gctagaagggt gaaatcccag aaaagtacgg tgaacactcc atcgaagttc	780
cagggtgctg caagttgtgt aatgctttga acgccttgcc aaaggaaaaa tgggctgtcg	840
ccacctctgg taccctgac atggccaaga aatggttcga cattttgaag atcaagagac	900

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cagaatactt catcaccgcc aatgatgtca agcaaggtaa gcctcaccca gaaccatact	960
taaagggtag aaacgggttg gggttcccaa ttaatgaaca agaccatcc aaatctaagg	1020
ttgttgctct tgaagacgca ccagctggta ttgctgctgg taaggctgct ggctgtaaaa	1080
tcgttggtat tgctaccact ttcgatttgg acttcttgaa ggaaaagggt tgtgacatca	1140
ttgtcaagaa ccacgaatct atcagagtcg gtgaatacaa cgctgaaacc gatgaagtcg	1200
aattgatctt tgatgactac ttatacgta aggatgactt gttgaaatgg taattttctt	1260
ttattttttt gataaaacta ctacgctaaa aataaaataa aaatgtatga ttccctcca	1320
ttccgacca attgtataat ttatatctg catgacttaa taatataata taatacttat	1380
aaaatacgaa tagaaaaatt taaacgatg taatgcaccc tttctttgt cgtcttcgga	1440
tgatctgcgc tgacaggtgg ttgcgcgaaa tcaagctggg ttagagaatt taacacagaa	1500
ataaaaaagg aagattcaat ctctgtttt gttttatata ttactataaa agtggttttt	1560
tttagtacga cgagaagtta gagtaattat aaaaggaatg cttattttaa tttatttctt	1620
agacttcttt tcagacttct tagcagctc agtttggtcc ttaacgacct tcttaacaat	1680
cttttggtct tcaatcaaga aagctctgac gattctttcc ttgacacagt tggcacatct	1740
ggaaccacgc taa	1753

<210> SEQ ID NO 4

<211> LENGTH: 1753

<212> TYPE: DNA

<213> ORGANISM: *Saccharomyces cerevisiae*

<400> SEQUENCE: 4

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cgatcatcat tgtgccccac aatccgoggc gtttccgtgt aagccgtcaa gtgaggactt	180
ttcgatgctt gaaagaaagt acgctcgga aaactacata gctgcccccc taaacggggc	240
tcccacgtga cgtaaagtag gaataataag aagccaagtc gtttcttttt attctaata	300
agttcgtttc ttttgatggt gtcattttca gaaatatata tatatgcgt taaatacaca	360
agctaaaaca acatagtttag gattgcaaaa ggtttctttt ctactcaatt tgggtctaact	420
cttttcatat taatagcgcc aaccagctag tgtttaccag atcagtggaa aaacataaaa	480
caataaaaac aatattcgga atgggattga ctactaaacc tctatctttg aaagttaacg	540
ccgctttggt cgacgtcgac ggtaccatta tcatctctca accagccatt gctgcattct	600
ggaggggatt cggttaaggac aaaccttatt tcgatgtga acacgttatc caagtctcgc	660
atggttgtag aacgtttgat gccattgcta agttcgctcc agactttgcc aatgaagagt	720
atgttaacaa attagaagct gaaattccgg tcaagtacgg tgaaaaatcc attgaagtc	780
cagggtgcagt taagctgtgc aacgctttga acgctctacc aaaagagaaa tgggctgtgg	840
caacttccgg taccgctgat atggcacaaa aatggttcga gcatctggga atcaggagac	900
caaagtactt cattaccgct aatgatgtca aacagggtaa gcctcatcca gaaccatct	960
tgaagggcag gaatggctta ggatatccga tcaatgagca agacccttcc aaatctaagg	1020
tagtagtatt tgaagacgct ccagcaggta ttgcccggc aaaagccgccc ggttgtaaga	1080
tcattggtat tgccactact ttgacttggt acttctctaa ggaaaaaggc tgtgacatca	1140
ttgtcaaaaa ccacgaatcc atcagagttg gcggctacaa tgccgaaaca gacgaagttg	1200
aattcatttt tgacgactac ttatatgcta aggacgatct gttgaaatgg taatctctca	1260

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aaatcgaaca tatttgagta ataattctca gatacagtc tttctatat tcgccacaaa 1320
acaagtaatg atgctaataa acgacacatt tataaaatca catcttattg attaaataaa 1380
tacgtagata gatttttttt tttaaaacat atagtgtgct attattctcg actctgtctc 1440
atctcagaaa aataaatgat aaaaaaggaa gtaaaatcct taaacgttat caggttatta 1500
gcaacttgaa agtgacagga gccacaacgg attaaaattt aatttctagt aaagaaatgt 1560
caagaagagt gggtatcaca ggattgggct gtgtaacgcc gttgggaaga tcattaagtg 1620
agtcatgggg gaatctgtct tcttccaaaa atggactcac accaatcaca tctttgccca 1680
actataatga ggactacaaa ctacagagaaa aaagtatccc atcaacgata acagtgggga 1740
agattccaga gaa 1753

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<210> SEQ ID NO 5
<211> LENGTH: 401
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TEF1 promoter mutant 2

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<400> SEQUENCE: 5

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tcgccgtacc acctcaaaag cccaagcgc agcataccaa atctcccctc tttcttctc 120
taggggtgca ctagtctact gtactaaggg ttgggggaag gagaaagaga ccgcctcgtc 180
ttcttttctt cgctgaaggg ggcaatagaa gtttttatca tgtctccttt ccttgagaac 240
cttttcttcg atcttgttct ctttcgacgg cctcccgctg gtatttaggt taatgaacgg 300
tcttcaacct ctcaagtttc agtttctctt ctcccgctct attacgacct ttcttacttc 360
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<210> SEQ ID NO 6
<211> LENGTH: 401
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TEF1 promoter mutant 7

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<400> SEQUENCE: 6

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ccgccgtacc acttcaaaac acccaagcac agcactacta attccccctc ctcttctctc 120
taggggtgca ttaattaccc gtactaaagg ttggaaaag gaaaagaga ccgcctcgtc 180
cctttttctt cgctcgagaa ggcaataaaa atttttatca cgtttcttct tcttgaaaac 240
tttttttctg attttgttct ctttcgacga cctccattg atatttgagt taacaaacgg 300
tcttcaattt ctcaagtttc agtttctctt tctctgttct attacaactt ttttacttc 360
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<210> SEQ ID NO 7
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: P9

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<400> SEQUENCE: 7

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cccaaggcag gacagttacc 20

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<210> SEQ ID NO 8
 <211> LENGTH: 21
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: P10

<400> SEQUENCE: 8

agcaccagat agagcaccac a 21

<210> SEQ ID NO 9
 <211> LENGTH: 21
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: P11

<400> SEQUENCE: 9

ggaccaccaa cagcacctag t 21

<210> SEQ ID NO 10
 <211> LENGTH: 21
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: P12

<400> SEQUENCE: 10

gtaagcaact gttgtttcag a 21

<210> SEQ ID NO 11
 <211> LENGTH: 401
 <212> TYPE: DNA
 <213> ORGANISM: *Saccharomyces cerevisiae*

<400> SEQUENCE: 11

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tcgccgtacc acttcaaaac acccaagcac agcatactaa atttccctc tttcttctc 120

taggggtgctg ttaattaccc gtactaaagg ttgggaaaag aaaaaagaga ccgcctcgtt 180

tcttttttctt cgctgaaaaa ggcaataaaa atttttatca cgtttctttt tcttgaaaat 240

tttttttttg atttttttct ctttcgatga cctccattg atatttaagt taataaacgg 300

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ttgctcatta gaaagaaagc atagcaatct aatctaagtt t 401

<210> SEQ ID NO 12
 <211> LENGTH: 62
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: P29

<400> SEQUENCE: 12

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cg 62

<210> SEQ ID NO 13
 <211> LENGTH: 60
 <212> TYPE: DNA
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: P30

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<400> SEQUENCE: 13

cgatgtctag ctcttcaatc atctccgga ggtcttccat gttttattta ggttctatcg 60

<210> SEQ ID NO 14

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: P33

<400> SEQUENCE: 14

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<210> SEQ ID NO 15

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: P34

<400> SEQUENCE: 15

aggcaacagg aaagatcaga gg 22

<210> SEQ ID NO 16

<211> LENGTH: 21

<212> TYPE: DNA

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 16

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<210> SEQ ID NO 17

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 17

tcaaaggcat tgcgatggtt 20

<210> SEQ ID NO 18

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 18

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<210> SEQ ID NO 19

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 19

ctctaacttc tcgtcgtagt 20

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<210> SEQ ID NO 20
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 20

caagcaggaa atccgtatca

20

<210> SEQ ID NO 21
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 21

tcatatggag caatcccact

20

<210> SEQ ID NO 22
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 22

caagtgagga cttttcggat

20

<210> SEQ ID NO 23
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 23

gtagtcaatc ccattccgaa

20

<210> SEQ ID NO 24
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 24

ggacgatctg ttgaaatggt

20

<210> SEQ ID NO 25
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: GPP deletion primer

<400> SEQUENCE: 25

cctgtccact ttcaagttgc t

21

<210> SEQ ID NO 26
<211> LENGTH: 391
<212> TYPE: PRT
<213> ORGANISM: *Saccharomyces cerevisiae*

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<400> SEQUENCE: 26

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 Ala Gly Arg Lys Arg Ser Ser Ser Ser Val Ser Leu Lys Ala Ala Glu
 20 25 30
 Lys Pro Phe Lys Val Thr Val Ile Gly Ser Gly Asn Trp Gly Thr Thr
 35 40 45
 Ile Ala Lys Val Val Ala Glu Asn Cys Lys Gly Tyr Pro Glu Val Phe
 50 55 60
 Ala Pro Ile Val Gln Met Trp Val Phe Glu Glu Glu Ile Asn Gly Glu
 65 70 75 80
 Lys Leu Thr Glu Ile Ile Asn Thr Arg His Gln Asn Val Lys Tyr Leu
 85 90 95
 Pro Gly Ile Thr Leu Pro Asp Asn Leu Val Ala Asn Pro Asp Leu Ile
 100 105 110
 Asp Ser Val Lys Asp Val Asp Ile Ile Val Phe Asn Ile Pro His Gln
 115 120 125
 Phe Leu Pro Arg Ile Cys Ser Gln Leu Lys Gly His Val Asp Ser His
 130 135 140
 Val Arg Ala Ile Ser Cys Leu Lys Gly Phe Glu Val Gly Ala Lys Gly
 145 150 155 160
 Val Gln Leu Leu Ser Ser Tyr Ile Thr Glu Glu Leu Gly Ile Gln Cys
 165 170 175
 Gly Ala Leu Ser Gly Ala Asn Ile Ala Thr Glu Val Ala Gln Glu His
 180 185 190
 Trp Ser Glu Thr Thr Val Ala Tyr His Ile Pro Lys Asp Phe Arg Gly
 195 200 205
 Glu Gly Lys Asp Val Asp His Lys Val Leu Lys Ala Leu Phe His Arg
 210 215 220
 Pro Tyr Phe His Val Ser Val Ile Glu Asp Val Ala Gly Ile Ser Ile
 225 230 235 240
 Cys Gly Ala Leu Lys Asn Val Val Ala Leu Gly Cys Gly Phe Val Glu
 245 250 255
 Gly Leu Gly Trp Gly Asn Asn Ala Ser Ala Ala Ile Gln Arg Val Gly
 260 265 270
 Leu Gly Glu Ile Ile Arg Phe Gly Gln Met Phe Phe Pro Glu Ser Arg
 275 280 285
 Glu Glu Thr Tyr Tyr Gln Glu Ser Ala Gly Val Ala Asp Leu Ile Thr
 290 295 300
 Thr Cys Ala Gly Gly Arg Asn Val Lys Val Ala Arg Leu Met Ala Thr
 305 310 315 320
 Ser Gly Lys Asp Ala Trp Glu Cys Glu Lys Glu Leu Leu Asn Gly Gln
 325 330 335
 Ser Ala Gln Gly Leu Ile Thr Cys Lys Glu Val His Glu Trp Leu Glu
 340 345 350
 Thr Cys Gly Ser Val Glu Asp Phe Pro Leu Phe Glu Ala Val Tyr Gln
 355 360 365
 Ile Val Tyr Asn Asn Tyr Pro Met Lys Asn Leu Pro Asp Met Ile Glu
 370 375 380
 Glu Leu Asp Leu His Glu Asp
 385 390

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<210> SEQ ID NO 27
<211> LENGTH: 440
<212> TYPE: PRT
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 27

Met Leu Ala Val Arg Arg Leu Thr Arg Tyr Thr Phe Leu Lys Arg Thr
 1             5             10            15
His Pro Val Leu Tyr Thr Arg Arg Ala Tyr Lys Ile Leu Pro Ser Arg
      20             25            30
Ser Thr Phe Leu Arg Arg Ser Leu Leu Gln Thr Gln Leu His Ser Lys
      35             40            45
Met Thr Ala His Thr Asn Ile Lys Gln His Lys His Cys His Glu Asp
 50             55            60
His Pro Ile Arg Arg Ser Asp Ser Ala Val Ser Ile Val His Leu Lys
 65             70            75            80
Arg Ala Pro Phe Lys Val Thr Val Ile Gly Ser Gly Asn Trp Gly Thr
      85             90            95
Thr Ile Ala Lys Val Ile Ala Glu Asn Thr Glu Leu His Ser His Ile
      100            105           110
Phe Glu Pro Glu Val Arg Met Trp Val Phe Asp Glu Lys Ile Gly Asp
      115            120           125
Glu Asn Leu Thr Asp Ile Ile Asn Thr Arg His Gln Asn Val Lys Tyr
      130            135           140
Leu Pro Asn Ile Asp Leu Pro His Asn Leu Val Ala Asp Pro Asp Leu
      145            150           155           160
Leu His Ser Ile Lys Gly Ala Asp Ile Leu Val Phe Asn Ile Pro His
      165            170           175
Gln Phe Leu Pro Asn Ile Val Lys Gln Leu Gln Gly His Val Ala Pro
      180            185           190
His Val Arg Ala Ile Ser Cys Leu Lys Gly Phe Glu Leu Gly Ser Lys
      195            200           205
Gly Val Gln Leu Leu Ser Ser Tyr Val Thr Asp Glu Leu Gly Ile Gln
      210            215           220
Cys Gly Ala Leu Ser Gly Ala Asn Leu Ala Pro Glu Val Ala Lys Glu
      225            230           235           240
His Trp Ser Glu Thr Thr Val Ala Tyr Gln Leu Pro Lys Asp Tyr Gln
      245            250           255
Gly Asp Gly Lys Asp Val Asp His Lys Ile Leu Lys Leu Leu Phe His
      260            265           270
Arg Pro Tyr Phe His Val Asn Val Ile Asp Asp Val Ala Gly Ile Ser
      275            280           285
Ile Ala Gly Ala Leu Lys Asn Val Val Ala Leu Ala Cys Gly Phe Val
      290            295           300
Glu Gly Met Gly Trp Gly Asn Asn Ala Ser Ala Ala Ile Gln Arg Leu
      305            310           315           320
Gly Leu Gly Glu Ile Ile Lys Phe Gly Arg Met Phe Phe Pro Glu Ser
      325            330           335
Lys Val Glu Thr Tyr Tyr Gln Glu Ser Ala Gly Val Ala Asp Leu Ile
      340            345           350
Thr Thr Cys Ser Gly Gly Arg Asn Val Lys Val Ala Thr Tyr Met Ala
      355            360           365
Lys Thr Gly Lys Ser Ala Leu Glu Ala Glu Lys Glu Leu Leu Asn Gly
      370            375           380

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Gln Ser Ala Gln Gly Ile Ile Thr Cys Arg Glu Val His Glu Trp Leu
 385 390 395 400

Gln Thr Cys Glu Leu Thr Gln Glu Phe Pro Leu Phe Glu Ala Val Tyr
 405 410 415

Gln Ile Val Tyr Asn Asn Val Arg Met Glu Asp Leu Pro Glu Met Ile
 420 425 430

Glu Glu Leu Asp Ile Asp Asp Glu
 435 440

<210> SEQ ID NO 28
 <211> LENGTH: 250
 <212> TYPE: PRT
 <213> ORGANISM: *Saccharomyces cerevisiae*

<400> SEQUENCE: 28

Met Pro Leu Thr Thr Lys Pro Leu Ser Leu Lys Ile Asn Ala Ala Leu
 1 5 10 15

Phe Asp Val Asp Gly Thr Ile Ile Ile Ser Gln Pro Ala Ile Ala Ala
 20 25 30

Phe Trp Arg Asp Phe Gly Lys Asp Lys Pro Tyr Phe Asp Ala Glu His
 35 40 45

Val Ile His Ile Ser His Gly Trp Arg Thr Tyr Asp Ala Ile Ala Lys
 50 55 60

Phe Ala Pro Asp Phe Ala Asp Glu Glu Tyr Val Asn Lys Leu Glu Gly
 65 70 75 80

Glu Ile Pro Glu Lys Tyr Gly Glu His Ser Ile Glu Val Pro Gly Ala
 85 90 95

Val Lys Leu Cys Asn Ala Leu Asn Ala Leu Pro Lys Glu Lys Trp Ala
 100 105 110

Val Ala Thr Ser Gly Thr Arg Asp Met Ala Lys Lys Trp Phe Asp Ile
 115 120 125

Leu Lys Ile Lys Arg Pro Glu Tyr Phe Ile Thr Ala Asn Asp Val Lys
 130 135 140

Gln Gly Lys Pro His Pro Glu Pro Tyr Leu Lys Gly Arg Asn Gly Leu
 145 150 155 160

Gly Phe Pro Ile Asn Glu Gln Asp Pro Ser Lys Ser Lys Val Val Val
 165 170 175

Phe Glu Asp Ala Pro Ala Gly Ile Ala Ala Gly Lys Ala Ala Gly Cys
 180 185 190

Lys Ile Val Gly Ile Ala Thr Thr Phe Asp Leu Asp Phe Leu Lys Glu
 195 200 205

Lys Gly Cys Asp Ile Ile Val Lys Asn His Glu Ser Ile Arg Val Gly
 210 215 220

Glu Tyr Asn Ala Glu Thr Asp Glu Val Glu Leu Ile Phe Asp Asp Tyr
 225 230 235 240

Leu Tyr Ala Lys Asp Asp Leu Leu Lys Trp
 245 250

<210> SEQ ID NO 29
 <211> LENGTH: 250
 <212> TYPE: PRT
 <213> ORGANISM: *Saccharomyces cerevisiae*

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<400> SEQUENCE: 29

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Met Gly Leu Thr Thr Lys Pro Leu Ser Leu Lys Val Asn Ala Ala Leu
1           5           10           15
Phe Asp Val Asp Gly Thr Ile Ile Ile Ser Gln Pro Ala Ile Ala Ala
20           25           30
Phe Trp Arg Asp Phe Gly Lys Asp Lys Pro Tyr Phe Asp Ala Glu His
35           40           45
Val Ile Gln Val Ser His Gly Trp Arg Thr Phe Asp Ala Ile Ala Lys
50           55           60
Phe Ala Pro Asp Phe Ala Asn Glu Glu Tyr Val Asn Lys Leu Glu Ala
65           70           75           80
Glu Ile Pro Val Lys Tyr Gly Glu Lys Ser Ile Glu Val Pro Gly Ala
85           90           95
Val Lys Leu Cys Asn Ala Leu Asn Ala Leu Pro Lys Glu Lys Trp Ala
100          105          110
Val Ala Thr Ser Gly Thr Arg Asp Met Ala Gln Lys Trp Phe Glu His
115          120          125
Leu Gly Ile Arg Arg Pro Lys Tyr Phe Ile Thr Ala Asn Asp Val Lys
130          135          140
Gln Gly Lys Pro His Pro Glu Pro Tyr Leu Lys Gly Arg Asn Gly Leu
145          150          155          160
Gly Tyr Pro Ile Asn Glu Gln Asp Pro Ser Lys Ser Lys Val Val Val
165          170          175
Phe Glu Asp Ala Pro Ala Gly Ile Ala Ala Gly Lys Ala Ala Gly Cys
180          185          190
Lys Ile Ile Gly Ile Ala Thr Thr Phe Asp Leu Asp Phe Leu Lys Glu
195          200          205
Lys Gly Cys Asp Ile Ile Val Lys Asn His Glu Ser Ile Arg Val Gly
210          215          220
Gly Tyr Asn Ala Glu Thr Asp Glu Val Glu Phe Ile Phe Asp Asp Tyr
225          230          235          240
Leu Tyr Ala Lys Asp Asp Leu Leu Lys Trp
245          250

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The invention claimed is:

1. A method of modifying a yeast cell for the production of ethanol, the method comprising:

reducing, but not eliminating, glycerol-3-phosphate dehydrogenase 1, Gpd1, protein activity in the yeast cell and eliminating glycerol-3-phosphate dehydrogenase 2, Gpd2, protein activity in the yeast cell; or

eliminating Gpd1 protein activity in the yeast cell and reducing, but not eliminating, Gpd2 protein activity in the yeast cell; or

reducing, but not eliminating, both Gpd1 and Gpd2 protein activity in the yeast cell,

wherein the reduction of Gpd1 protein activity and/or Gpd2 protein activity is achieved by reducing the expression of the GPD1 gene and/or the GPD2 gene by at least 50% compared to the expression of the GPD1 gene and/or the GPD2 gene in the unmodified wild type yeast cell and wherein the modified yeast cell has equivalent growth to that of an unmodified yeast cell under oxygen-limited conditions and an increased yield of ethanol.

2. The method as recited in claim 1, wherein the GPD1 gene and/or the GPD2 gene is expressed by a weak promoter

that is operably linked to the GPD1 gene or to the GPD2 gene, wherein the weak promoter causes less than or equal to 20% transcription of the TEF1 promoter operably linked to the GPD1 gene or the GPD2 gene.

3. The method as recited in claim 2, wherein the weak promoter operably linked to the GPD1 gene or the GPD2 gene is a promoter according to SEQ ID NO 5 or SEQ ID NO 6.

4. The method as recited in claim 1, wherein the activity of the glycerol-3-phosphatase 1, Gpp1, protein and/or the glycerol-3-phosphatase 2, Gpp2, protein, is additionally reduced in the yeast cell, comprising:

reducing Gpp1 protein activity in the yeast cell and eliminating Gpp2 protein activity in the yeast cell; or eliminating Gpp1 protein activity in the yeast cell and reducing Gpp2 protein activity in the yeast cell; or reducing both Gpp1 protein and Gpp2 protein activity in the yeast cell,

wherein the reduction of the activity of the Gpp1 protein and/or the Gpp2 protein is achieved by reducing the expression of the GPP1 and/or the GPP2 gene by at least 50% compared to the expression of the GPP1 gene and/or the GPP2 gene in a wild type yeast cell.

5. The method as recited in claim 4, wherein the GPP1 gene and/or the GPP2 gene is expressed by a weak promoter that is

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operably linked to the GPP1 gene or the GPP2 gene, wherein the weak promoter causes less than or equal to 20% transcription of the TEF1 promoter operably linked to the GPP1 gene or the GPP2 gene.

6. The method as recited in claim 5, wherein the promoter operably linked to the GPP1 gene or the GPP2 gene is a weak promoter according to SEQ ID NO 5 or SEQ ID NO 6.

7. A method for the production of ethanol, the method comprising

providing a yeast cell modified as recited in claim 1; providing biomass; and

growing the modified yeast cell in a presence of the biomass under conditions that allow for a production of ethanol.

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